C. POTENTIOMETRIC ANALYSIS OF PURE ANIMAL PROTEINS.

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IN previous articles one of the authors has published a series of experimental results obtained with animal and vegetable proteins by two different methods: potentiometric titration, and analysis by means of the dielectric constant [Errera, 1932, 1, 2; Errera and Bloch, 1932; Errera *et al.*, 1932].

In the experiments which form the basis of the present article, the authors had as their object the systematic study of some pure animal proteins with a view to the elucidation of the following points.

- (1) The determination of the isoelectric points of these pure proteins.
- (2) The stoichiometric relationship of the combinations of these proteins with acids and bases.
- (3) The value of the acid and basic equivalents of these proteins.
- (4) The acidic and basic dissociation constants of the proteins.
- (5) The determination of the number of COOH and NH₂ groups for each of the molecules studied.

EXPERIMENTAL METHODS AND PROCEDURE.

The method of analysing a solution by potentiometric titration is well known [see *e.g.* Muller, 1932].

In order to overcome the difficulties encountered in the estimation of the p_H in the case of proteins, we have used antimony and glass electrodes.

In the previous work, only the antimony electrode was used. In the present experiments we have used either a glass electrode alone, or else we have used a glass electrode and checked the results obtained against an antimony electrode.

The use of an antimony electrode necessitates the employment of only slight precautions during the measurement; no gas is needed, and equilibrium is fairly rapidly attained in the case of protein solutions¹. Antimony electrodes are made by melting metal of a high degree of purity in a graphite crucible under carbon monoxide. The cell in which they are to be employed is built up on the following principles: saturated calomel electrode—solution of unknown p_H —antimony electrode. The standard curve for the antimony electrode was determined with the aid of a buffer solution to which white of egg had been added. This curve consists of three straight lines joined together by very flat curves. The straight line portions of the curve may be held to correspond to three different conditions of equilibrium of the Sb in acid, neutral and alkaline media respectively.

¹ We wish to express our thanks to Mr H. Vogels for the method of carrying out the potentiometric measurements with an antimony electrode. For the details of this method, see Vogels [1930], and also the work which Mr Vogels will shortly publish.

The glass electrode furnishes more reliable results because in this case the equilibrium is unique, and also because a little antimony always dissolves in the solution. The experimental set-up is in many respects similar to that used by one of the authors in previous experiments on the measurements of p_H in blood-plasma [Errera *et al.*, 1930]. An additional interrupter permits of working alternatively with a glass and with an antimony electrode. The concordant results obtained lead to the conclusion that neither the solution of traces of antimony nor the formation of a layer of adsorbed proteins on the surface of the glass electrode vitiates the results.

Once the results are obtained, the usual procedure is to construct a graph, using the number of cc. of the reagent added as abscissae, and the corresponding p_H values as ordinates. If however this method is adopted in the case of proteins, the resulting curves do not show the characteristic points with sufficient clearness.

Fig. 1 shows the titration curve for pig-nut globulin dissolved in NaOH. The curve shows three characteristic points of which two correspond to points of maximum inclination of slope while the third, which lies between the other two, corresponds to a point of minimum inclination. The significance of these points will be discussed later.

Fig. 1.

Fig. 2.

In order to bring out the experimental results more clearly, we have chosen another mode of representation: as abscissae are plotted the p_H values, and as ordinates the difference in millivolts between two¹ succeeding p_H values (Δp_H) corresponding to an addition of a constant volume of the titrating reagent. Thus for instance alkaline globulin is titrated with $N/10$ HCl, 0.5 cc. is added each time and after each measurement of the resulting p_H the difference between this and the preceding value is plotted on the graph.

Fig. 2 shows the same results as those used to construct Fig. 1, plotted according to this new method. On comparing the two curves, we see that the

¹ The millivolt differences are calculated with respect to the final p_H .

two maxima in Fig. 2 correspond to the two points of maximum tangential inflection in Fig. 1, while the minimum in Fig. 2 coincides with the point of minimum tangential inflection. It is at once obvious that the graph based on the differential representation brings out the characteristic points much more clearly.

The question now arises as to the significance of the maxima and minimum of Fig. 2. The two maxima represent the neutralisation of one component by the titrating reagent (for instance base with acid). The minimum represents the isoelectric point.

To the left of the first maximum and to the right of the second maximum the reaction proceeds independently of the protein. The first maximum represents the neutralisation of the free base and the beginning of the reaction between acid and the COONa group of the protein.

When we arrive at the isoelectric point, all the COONa groups have been converted into COOH groups by the acid, and the combination of the amino-groups of the protein with the acid now begins. At the second maximum this combination is complete, and the reaction of the free acid upon the protein begins.

In these experiments we have further used reagents of different valency: hydrochloric and sulphuric acids as acid reagents, sodium hydroxide and barium hydroxide as basic reagents. The choice of the concentration of the titrating reagent in relation to that of the protein to be titrated should only be made after carrying out preliminary investigations.

When hydrochloric acid is titrated with sodium hydroxide, a normal curve is obtained whose differential representation gives only a single maximum. If the titration is now reversed, and carried out in the presence of CO_2 from the air, two maxima are observed. This is due to the presence of NaHCO_3 which continues to form during the titration in the air, potentiometric titrations being extremely sensitive to small traces of this substance. This presence of bicarbonate may indeed be the cause of certain errors in the determination of the isoelectric point when this lies close to the jump in potential due to the bicarbonate. For this reason we have modified the method used in our previous experiments as follows: two portions of the isoelectric protein were titrated, the one with an acid and the other with an alkaline reagent, the correction to be made after comparison with the blank being further taken into account where necessary [see Errera, 1932, 1, 2; Errera *et al.*, 1930; 1932; Harris, 1923].

PREPARATION OF THE PROTEINS.

(a) *Egg-albumin*. The albumin was separated from the other proteins contained in beaten white of egg by half saturating with ammonium sulphate. The globulins thus precipitated were separated by filtration, and the albumin was precipitated by complete saturation of the filtrate with a slight excess of powdered ammonium sulphate. The albumin was then dialysed through a cellophane membrane, the water outside the membrane being changed until no reaction could be detected for the sulphate ion. Subsequently the albumin was purified by electrodialysis according to the method of Pauli and Weiss [1928] through a nitrocellulose membrane.

(b) *Serum-albumin*. Albumin from dog-serum was separated from the globulin by half saturating with ammonium sulphate at 37° . After filtration, the albumin was precipitated from the filtrate by complete saturation with a slight excess of ammonium sulphate, also at 37° . The purification of the albumin was subsequently carried out in the same way as for egg-albumin.

(c) *Haemoglobin*. This was prepared from horse blood according to the method of Svedberg and Nichols [1927] and subsequently purified by electro-dialysis.

(d) *Caseinogen*. Very pure caseinogen prepared by the method of Hammarsten (Kahlbaum) was subsequently subjected to electro-dialysis.

EXPERIMENTAL RESULTS.

(1) *Isoelectric point.*

The isoelectric point of each protein was determined by the potentiometric measurement of the p_H of the protein after purification by dialysis and electro-dialysis controlled by conductivity measurements. We have found that proteins prepared in this manner are always at the isoelectric point, irrespective of whether the membrane used for electro-dialysis is of nitrocellulose or of parchment.

Table I.

Protein	No of experiments	Limits of p_H of the isoelectric point	Mean of preceding column
Egg-albumin	25	4.7-4.85	4.75
Serum-albumin	15	5.4-5.5	5.45
Haemoglobin	15	6.5-6.6	6.55
Caseinogen	15	5.6-5.8	5.7

We believe that we are justified in drawing from the above results the conclusion that this method of determining the p_H of a protein purified by electro-dialysis is rational and gives accurate results, at any rate in so far as the determination of the isoelectric point of simple proteins is concerned.

As we have already explained, portions of the isoelectric protein were titrated respectively with an acid and with a base. In most cases however we also titrated back: thus after having first titrated with an acid, the same solution was subsequently titrated back with a base. The curve obtained had the form shown in Fig. 2, and the isoelectric point corresponding to the minimum was thus determined again by this second method. The resulting agreement was excellent, except for small variations in certain cases, which could be traced to the interfering presence of bicarbonate.

(2) *Stoichiometric relationship of the combination of the proteins with acids and bases.*

This relationship can be found to a first approximation by means of potentiometric titration, provided care is taken not to work in a too highly alkaline medium (see previous publications). In the present experiments we have been at particular pains to establish the stoichiometric relationship starting from simple proteins purified by electro-dialysis. To this end titrations were carried out on separate portions, some of which were titrated with mono- and di-basic acids, while others were titrated with a uni- and a bi-valent base. The concentration of the titrating reagent in the experiments shown in Tables II-V, varied from $N/10$ to $N/100$ according to the concentration of the protein to be titrated. In the Tables the number of cc. used have been uniformly calculated to correspond to a concentration of $N/100$. This number of cc. corresponds to the quantity of reagent fixed by the protein and is represented in Fig. 2 by the passage of the curve from the isoelectric point to one of the two maxima.

Table II.

Pure protein in water	Quantity of protein g.	Number of cc. NaOH	Same calc. for 1 g. protein	Number of cc. HCl	Same calc. for 1 g. of protein
Egg-albumin	2.7	73	27.0	15	5.5
	0.9	25	27.7	5	5.5
	0.45	13	28.9	2.5	5.5
	0.24	7	29.2	1.5	5.5
Serum-albumin	0.88	16	18.2	6.5	7.4
	0.44	8.5	19.3	3.5	7.9
	0.22	4	18.2	1.75	7.9
	0.09	1.6	17.8	0.75	8.3
Haemoglobin	0.45	7	15.6	5	11.1
	0.34	5	14.7	3.5	10.3
	0.23	3.2	14	2.5	10.8

Table III.

Protein dissolved in NaOH	Quantity of protein g.	Number of cc. HCl used between the two maxima	Same calc. for 1 g. of protein
Caseinogen	0.5	80	16
	0.2	28	14
	0.1	16	16
	0.05	8	16

Table IV.

Pure protein in water	Quantity of protein g.	Number of cc. Ba(OH) ₂	Same calc. for 1 g. of protein
Egg-albumin	0.9	26	28.8
	0.6	17.5	29
	0.3	8	27
Haemoglobin	0.45	7.5	16.6
	0.34	5	14.7
	0.23	3.5	15.2

Table V.

Pure protein in water	Quantity of protein g.	Number of cc. H ₂ SO ₄	Same calc. for 1 g. of protein
Egg-albumin	1.2	6.5	5.4
	0.9	4.6	5.1
	0.6	3.5	5.8

The column in which the number of cc. of reagent used is calculated for 1 g. of protein shows the stoichiometric relationship of the reaction between the protein and the acids and bases employed in terms of the concentrations of the proteins and the titrating reagent and also of the valency of the latter.

(3) Taking as a basis the above Tables, we have calculated the quantity of each protein expressed in g., which is needed to fix one g.-ion of H⁺ or one g.-ion of OH⁻.

Table VI shows the results.

Table VI.

Protein	Gram-equivalent of protein fixing one g.-ion of OH ⁻	Gram-equivalent of protein fixing one g.-ion of H ⁺
Egg-albumin	3420	18000
Serum-albumin	5450	12900
Haemoglobin	6800	9200
Caseinogen	860	5000

(4) The fourth point which we have studied is the calculation of the dissociation constants of the pure proteins, when viewed as acids (K_a) or when viewed as bases (K_b), starting from the p_H corresponding with half-titrations (see previous publications). These values are of course only mean values, for it is probable that the different COOH and NH_2 groups do not dissociate at the same p_H , since they are situated differently in the protein molecule (compare for example the different dissociation of the various H^+ ions in the case of phosphoric acid). But the very fact that the form of the curve attained between the minimum and each of the two maxima is continuous, allows one to carry out the calculations: for it is obvious that otherwise the curve should present a series of small intermediary maxima corresponding with the various active groups.

Table VII shows the mean values obtained, together with the number of titrations on which they were based.

Table VII.

Protein	No. of titrations	Mean K_a	No. of titrations	Mean K_b
Egg-albumin	15	3.6×10^{-7}	10	2.8×10^{-11}
Serum-albumin	10	5.5×10^{-8}	10	2.7×10^{-10}
Haemoglobin	8	1.2×10^{-8}	6	7.6×10^{-10}
Caseinogen	5	5.0×10^{-8}	5	3.0×10^{-10}

(5) The fifth point is the determination of the number of COOH and NH_2 groups per molecule of protein, it being of course understood that these molecules have the dimensions of colloid particles. This calculation can be carried out if the acid and basic equivalents of the proteins (shown in Table VI) are known, and also their molecular weights. For these latter we have adopted those found by Svedberg and his collaborators.

Table VIII gives the numbers of these COOH and NH_2 groups which act as acids and bases capable of dissociation.

Table VIII.

Protein	Molecular weight	No. of COOH groups	No. of NH_2 groups
Egg-albumin	34,500	10	1.9
Serum-albumin	67,500	12.3	5.2
Haemoglobin	68,000	10	7.4
Caseinogen	98,000	114	19.6

EXPERIMENTS UNDERTAKEN AS A CHECK¹.

In order to establish the particular character of the chemical combinations of proteins, some experiments were carried out on inert adsorbent substances. Freshly precipitated $BaSO_4$, activated charcoal and silica which had been prepared by an industrial process of electro-osmosis were studied. In all three cases the potentiometric curves were similar to that obtained with pure water: and it is therefore impossible for the curves observed with proteins, except perhaps in a very small degree, to be due to adsorption phenomena.

¹ It may be noted in passing that the experiments carried out on albumins seem to indicate that with increasing time, even in a medium of pure water, a portion of the albumin is transformed into another protein having a different and less acid isoelectric point. Further experiments are however needed.

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